Analysis of PTEN, estrogen receptor α and progesterone receptor expression in endometrial hyperplasia using tissue microarray

Beata Pieczyńska, Szymon Wojtylak, Anton Żawrocki, Wojciech Biernat

Department of Pathology, Medical University of Gdańsk, Poland

We compared the immunohistochemical expression of PTEN, estrogen receptor α (ER-α) and progesterone receptor (PR) in a series of endometrial hyperplasia (EH) and in disordered proliferative endometrium (DPE) by tissue microarray (TMA). The material consisted of 141 endometrial curretings including 98 cases (study group) diagnosed as EH [59-simple (SH), 20-complex (CH), 19 atypical (AEH)] and 43 cases (control group) with DPE due to anovulation. The mean *PTEN* expression index decreased in order DPE-EH-AEH groups ($p < 0.05$). The mean value of the ER-α index increased in order DPE-SH-CH and decreased in AEH group, whereas the PR expression index decreased in order DPE-EH-AEH ($p > 0.05$ and $p > 0.05$, respectively). These results show that steroid hormone receptor status influences the architectural changes of endometrium rather than cytological ones. On the other hand, decreased PTEN expression correlates more closely with the cytological atypia in endometrial cells. In our opinion ER-α and PR may be useful markers predicting therapy response in EH. PTEN presents as a strong prognosticator which may help in determining the risk of progression in advanced stages of EH, especially those with atypical cytological features.

Key words: PTEN, estrogen receptor, progesterone receptor, endometrial carcinogenesis, immunohistochemistry.

Introduction

Endometrial carcinoma (EC) is the most common malignancy of the female genital tract. Based on morphology, molecular data, clinical course and prognosis, two different types of endometrial carcinoma were distinguished and referred to as type I and type II uterine corpus carcinomas. Development of the former group depends on hyperestrogenism, chronic anovulatory cycles, obesity, hypertension, diabetes mellitus, hyperlipidemia and endometrial hyperplasia. These tumours usually present with endometrioid or mucinous morphology. According to the Westin model, type I endometrial carcinoma has frequent *PTEN*, *PIK3CA*, and *KRAS* gene mutations and microsatellite instability (MSI). *PTEN* mutations occur most frequently. This gene (Phosphatase and Tensin homologue deleted from chromosome 10) is located on chromosome 10q23.3 and functions as a tumour suppressor gene. Its protein product inhibits PI3K/AKT pathway and it may result in increased cell proliferation. *PTEN* inactivation may be a consequence of somatic mutations (up to 83% cases of EC), germline mutations (e.g. in Cowden syndrome), loss of heterozygosity (40% cases of EC) and promoter hypermethylation (20% cases of EC). *PTEN* mutations have also been observed in 15-55% of endometrial hyperplasia (EH), a condition considered to be a precursor lesion for type I endometrial carcinoma [1-3]. Some previous studies postulated the role of *PTEN* alterations as a strong prognosticator determining the risk of progression in individual cases of endometrial hyperplasia [4, 5].

The role of steroid hormone receptors (ER and PR) in the pathogenesis and therapeutic response of EH and type I endometrial carcinoma has been investigated previously [6-12]. We undertook this study to analyze the expression of PTEN protein and the steroid hormone receptors in different types of EH and in DPE (disordered proliferative endometrium) due to anovulation. Moreover, the aim of the study was to find out whether the analyzed proteins correspond more strongly with the architectural abnormalities (complexity of glands) or with the atypical features.

Material and methods

We analyzed endometrial curretings from 141 patients diagnosed in the Institute of Obstetrics and Female Diseases, Medical University of Gdańsk between 1994 and 2001. The study group consisted of 98 cases of EH, including 79 cases of SH and 19 cases of atypical EH (AEH). As the control group, 43 cases of DPE were selected. To the control group (disordered proliferative endometrium) we selected cases diagnosed strictly according to the criteria proposed by Mazur and Kurman [13]. These cases presented a pattern with few focally dilated glands confined to the endometrial functionalis. The rest of the glands presented a morphology typical of normal proliferative endometrium (tubular or slightly tortuous). The samples were obtained from patients at the perimenopausal age (mean 47.8 years in the EH group and 45.5 years in the DPE group). Endometrial curretings were formalin-fixed and paraffin-embedded. They were cut and stained with HE for the purpose of the study. Paraffin blocks were used for formation of tissue microarrays. Diagnosis of endometrial hyperplasia was rendered by two pathologists according to criteria of the WHO classification [14].

For tissue microarrays, the most representative histologic areas were selected from the routinely stained slides. In each case, 3 tissue cores (1.5 mm in diameter) were punched out from the paraffin ('donor') blocks containing primary endometrial curretings and transferred into a recipient paraffin block using Manual Tissue Arrayer (MTA 1, Beecher Instrument Inc.). Finally, each of the tissue microarray recipient blocks consisted of 36 cores (included 12 cases × 3 cores each). The tissue microarray paraffin blocks were cut at 4 μm histological slides and immunohistochemical stains were performed.

Immunohistochemistry

Immunohistochemical studies were performed with the following monoclonal antibodies: PTEN (Novocastra, NCL-PTEN), ERα (DAKO), PR (DAKO) using Visualization System: Novolink™ Polymer Detection System.

Tissue microarray sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval procedure was performed using Target Retrieval Solution citrate pH 6.0 (DAKO, Glostrup, Denmark) for 8.5 min in an electric pressure cooker and cooling afterwards for 20 minutes before immuno-staining. Then, the slides were exposed to 3% hydrogen peroxide solution (cat. No. RE7101) to block endogenous peroxidase and Protein Block (cat. No. RE7102) for 5 minutes each. The primary monoclonal antibodies were incubated at dilutions of 1 : 200 (ER-α and PR) and 1 : 800 (PTEN) for 1.5 hours at room temperature. Next, Post Primary Block (Novocastra) and NovoLink Polymer (Novocastra) were used for 30 min each. To visualize the reaction, we used 3,3'-diaminobenzidine (DAB) as a chromogen and counterstained slides with haematoxylin for 5 minutes each. Between each of the steps, the slides were washed with phosphate buffered solution (PBS).

As the immunohistochemical staining procedures resulted in loss of some TMA sections, only those cases with two or more cores left were included into the analysis. For an objective assessment of protein expression, the compound H-score index, including intensity of reaction and percentage of positive cells was used. The intensity of the immunostaining was graded from 0 to 3: 0 – no reaction, 1 – weak reaction, 2 – moderate reaction, 3 – strong reaction. The reference point for the intensity of staining was immunoreactivity of stromal cell nuclei. The percentage of positive nuclei was measured in 1000 cells. The final H-score was calculated as below:

$$\text{H-score} = 3 \times (\% \text{ of cells}) + 2 \times (\% \text{ of cells}) + 1 \times (\% \text{ of cells}).$$

Therefore, the final index score ranged from 0 to 300 points. Designated indices for each case were used to calculate the mean index for the analyzed groups. For statistical analysis, the U-Mann-Whitney test with a significance of $p < 0.05$ was used. All statistical calculations were performed in Statistica 8.0 software.

Results

The mean patients' age at the time of primary diagnosis was 47.8 and 45.5 in the study and the control group, respectively (no statistical significance).

PTEN

PTEN expression was present both in the nuclei of endometrial and stromal cells. It was revealed in 41 cases of proliferative endometrium (41/42, 97.6%), in 71/74 (96%) cases in a group of endometrial hyperplasia and 15/16 (93.7%) cases of hyperplasia with atypia. The cellular immunoreactivity was heterogeneous. Some cells within an endometrial gland or glands did not reveal PTEN expression either in atypical hyperplasia or in DPE and EH. Expression observed in the DPE group was stronger and more diffuse compared to endometrial hyperplasia. Atypical endometrial hyperplasia presented pre-

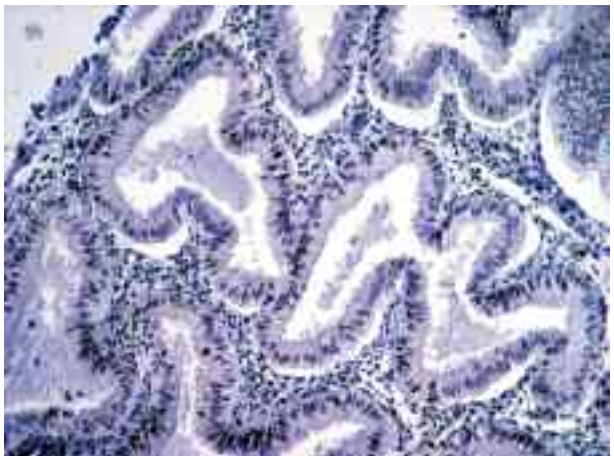

Fig. 1. Irregular nuclear staining with PTEN in glandular cells observed in atypical hyperplasia (some nuclei are PTEN null)

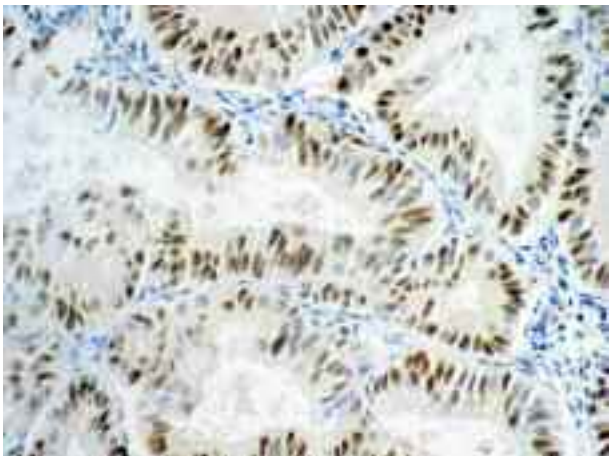

Fig. 2. Estrogen receptor α expression in atypical hyperplasia. Irregular nuclear staining is presented in glandular cells

dominantly weak (1+) and diffuse immunoreactivity or irregular expression with various numbers of negative cells (Fig. 1). Results of PTEN expression in analyzed groups are shown in Table I.

Mean H-score of PTEN expression decreased significantly in the following order: DPE > hyperplasia > AEH groups.

Estrogen receptor α and progesterone receptor status

Nuclear positive staining for ER-α was present in 128 from 131 analyzed cases (Fig. 2). Results of mean H-score expression of ER-α are shown in Table II.

The expression of ER-α increased from DPE to EH and decreased in AEH. Moreover, the highest level of index values was observed in complex hyperplasia. The statistical analysis did not indicate significant differences among analyzed groups.

Progesterone receptor expression was detected in all analyzed cases except for one case of AEH. In DPE, nuclear PR immunoreactivity was strong and diffusely distributed in the endometrial cells. The intensity of reaction decreased in EH and AEH (Fig. 3). The mean value of H-score for progesterone receptor is presented in Table III.

The average index values of PR differed between the following groups: DPE, EH and AEH but the mean val-

Table I. Values of H-score index for PTEN

	PTEN			
	N	POSITIVE N (%)	NEGATIVE N (%)	MEAN H-SCORE
DPE	42	41 (97.6)	1 (2.4)	176.6
EH	74	71 (96)	3 (4)	111.4
AEH	16	15 (93.7)	1 (6.3)	67.9

DPE – disordered proliferative endometrium, EH – endometrial hyperplasia, AEH – atypical endometrial hyperplasia

Table II. Values of H-score index for ER-α

	ER-α			
	N	POSITIVE N (%)	NEGATIVE N (%)	MEAN H-SCORE
DPE	41	40 (97.6)	1 (2.4)	**130.3**
EH	75			**150.05**
Simple	58	57 (96.6)	1 (1.7)	145.4
Complex	17	17 (85)	0	154.7
AEH	15	14 (93.3)	1 (6.7)	**136.4**

DPE – disordered proliferative endometrium, EH – endometrial hyperplasia, AEH – atypical endometrial hyperplasia, ER-α – estrogen receptor α

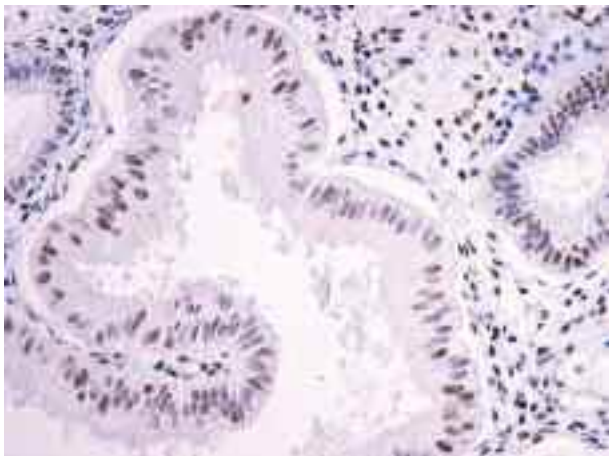

Fig. 3. Progesterone receptor expression in atypical hyperplasia presenting as irregular, predominantly weak nuclear staining in glandular cells

ue of H-score did not show significant differences. The ratio of PR expression tended to be lower in the AEH group in relation to the other two analyzed groups.

Discussion

Development of type I endometrial carcinoma is a sequential, long-term process dependent upon an unopposed estrogen stimulation and preceded by endometrial hyperplasia. Molecular alterations underlying the malignant transformation of endometrial cells include mutations in *PTEN*, *PIK3CA*, *KRAS*, *CTNNB1* (for β-catenin) genes and microsatellite instability [2]. *PTEN* inactivation, being the most frequent, occurs in up to 83% of endometrioid carcinomas and in 15-55% of endometrial hyperplasias with or without atypia. Most of these cases are due to sporadic mutations in the *PTEN* gene [2, 5].

However, the results of immunohistochemical analysis of PTEN expression in endometrial hyperplasia are ambiguous and vary across published studies. There are several factors that cause such disparities.

The first problem stems from the inconsistent classification systems of endometrial hyperplasia. Some authors compared PTEN expression to hyperplasia classification based on the endometrial intraepithelial neoplasia (EIN) terminology [4, 5, 15]. PTEN expression was lost in lesions defined as EIN or EEC compared to benign hyperplasia and normal proliferative endometrium. Baak *et al.* [4] demonstrated that PTEN loss is predictive for progression to carcinoma in morphometrically-defined EIN cases with D-score < 1. They presented positive (50%) versus negative (100%) predictive value with the use of this combined method. Studies based on selecting the EIN-type lesions demonstrated PTEN loss as a potential prognostic marker useful in evaluating cases with a high risk of progression to carcinoma. Contrary to EIN scheme, others analyzed PTEN activity according to the WHO classification of endometrial hyperplasia [2, 16, 17]. However, the prognostic value of such an approach was put in doubt by Baak *et al*. [4]. Another reason for inconsistent results is the relatively small case series (as demonstrated in a review by Allison *et al*. [18]). The aim of our study was to verify the above-mentioned inconsistencies by comparing the PTEN expression in a relatively large sample of endometrial hyperplasia cases ($n = 98$) subdivided according to the 1994 WHO classification scheme.

The second problem is a result of inconsistent methods used in quantification of PTEN expression [19]. Some authors interpret "PTEN loss" only if the entire gland or glands do not express the protein (so-called PTEN null glands) [4, 5]. They have observed that the percentage of cases with PTEN null glands increases in a stage in progression from normal endometrial proliferation to simple hyperplasia and EIN. Whereas Cirpan *et al*. [15] have revealed that visible differences between EIN, EEC and proliferative endometrium are associated with partial loss of PTEN but not with complete loss. Sarmadi *et al*. [20] used a four-grade system of PTEN immunoreactivity evaluation. They analyzed EH and endometrial carcinoma separating the cases into grade 0 (lack of reaction), +1 (weak intensity), +2 (moderate intensity), and +3 (strong intensity). Kapucuoglu *et al*. [16] used the H-score to estimate PTEN expression in EH and EC. We adopted this method because it seems to be the most objective and also indispensible for eval-

Table III. Values of H-score index for PR

	PR			
	N	POSITIVE N (%)	NEGATIVE N (%)	MEAN H-SCORE
DPE	39	39 (100)	0	**223.9**
EH	75			**178.7**
Simple	56	56 (100)	0	161.2
Complex	19	19 (100)	0	196.2
AEH	18	17 (94.4)	1 (5.6)	**161.7**

DPE – disordered proliferative endometrium, EH – endometrial hyperplasia, AEH – atypical endometrial hyperplasia, PR – progesterone receptor

uation of the heterogeneous pattern of PTEN expression we observed in the studied lesions.

All of the above-mentioned limitations in the interpretation of the disturbances of PTEN expression are complicated by the fact that early events of its inactivation are observed in the histologically normal endometrium in up to 43% of cases [21]. This phenomenon suggests a potential role of PTEN inactivation as an early latent precancer in endometrial carcinogenesis. Moreover, Lacey *et al*. [22] have observed that prevalence of PTEN null glands is similar in EH and in the normal endometrium in pre-menopausal women. This result was based on a comparative analysis of 104 index endometrial biopsies with available hysterectomy specimens. In 38% of those cases, PTEN null glands were not seen in the post-hysterectomy specimen. According to these observations, it could be concluded that the interpretation of areas in endometrium with PTEN loss is controversial and must be done carefully.

Besides the different methods of PTEN IHC scoring system, the control group included also varied in different reports. Both Sarmadi *et al*. [20] and Kapucuoglu *et al*. [16] used samples of endometrial proliferation typical of a physiological cycle as a control group. In other studies, the so-called normal endometrium is not precisely described. In our opinion, it is more appropriate to compare the study group with the endometrial biopsies from the anovulatory cycles. Such an approach provides the tissue with functional alterations remaining in close proximity to pathological states in progression to endometrial carcinoma and enables an evaluation of them as a continuum. The mean whole index of PTEN expression in our study significantly decreased starting from DPE to EH and AEH. This indicates that the loss of PTEN activity increases accordingly with architectural changes in the endometrium and cumulates most predominantly in atypical lesions. However, we must be careful in the interpretation of our results of PTEN expression. The antibody used in our study (NCL-PTEN, Novocastra, clone 28H6) was presented by Pallares *et al*. [23] as producing a non-specific staining. Therefore, the results of our study should be amended by a correlation with the expression of other proteins regulated by PTEN, including those involved in the PI3/AKT pathway, BUD protein and/or genetic testing for PTEN-encoding region on 10q23.

The significance of hormone receptor expression in development and therapeutic response of endometrioid carcinoma has been recently recognized. Most authors cited here used mean percentage of positive cells as a scoring system in their immunohistochemical analysis of the hormonal receptor status, thus making it easier to compare their findings [9, 11, 24, 25].

There are a few studies presenting the trend of an increasing ER-α index from DPA to SH and CH with a noticeable decrease in AEH and EC groups [6, 8, 9, 25]. Meanwhile, completely opposite results were published by Bircan *et al.* [24], who observed that ER-α index is the highest in the AEH group. In addition, Nunobiki *et al*. [11] analyzed a noteworthy group (n = 60) of atypical hyperplasia cases and similarly to Bircan *et al*. obtained highest levels of ER-α in AEH.

In our study, the mean ERα index increased in the following order DPE < SH < CH and decreased in the AEH group. The highest level of ER-α index was expressed in the complex hyperplasia group, indicating that it correlates much more with architectural (complexity of glands) than with severity of nuclear abnormalities (atypia). These observations suggest an important role of ER-α in the development of endometrial hyperplasia. Our results of the expression of ER-α in DPE and EH supported the observations presented in most of papers discussed above. In our opinion, estrogen overstimulation is reflected by architectural disorder rather than cytological changes seen in hyperplasia.

The mean levels of PR decreased in the following order DPE > EH > AEH groups, comparably to the findings of Nunobiki *et al.* [11]. The lowest mean level of PR expression occurred in the group of AEH. Moreover, PR immunoreactivity observed in AEH was the most heterogeneous compared to DPE and EH groups. The vast majority of AEH cases presented with an increased number of PR-negative cells. These observations suggest that AEH lesions may be less responsive to antiestrogen therapy with progestins.

Summarizing, development of type I endometrial carcinoma is a multifactorial process. The analyzed markers play important but different roles in pathogenesis and development of this neoplasm and its precursors. Eestrogen receptor α and PR status seems to correlate with architectural changes of the endometrium, whereas the loss of PTEN expression more closely reflects cytological abnormalities in endometrial hyperplasia. These observations may suggest hormonal receptors as strong predictors of therapeutic response, whereas PTEN as a useful prognosticator of the risk of progression for selected cases of endometrial hyperplasia, especially those with atypical histologic features.

Acknowledgement

This work was supported by grant W-156 from Medical University of Gdańsk.

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Address for correspondence

Szymon Wojtylak MD, PhD
Department of Pathology
Medical University of Gdańsk
ul. Dębinki 7
80-211 Gdańsk
phone: +48 58 349 16 50
e-mail: swojtylak@gumed.edu.pl